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Pharmacognostical Evaluation of *Zanthoxylum nitidum* Bark

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Abstract: *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae), called *Tez-mui* in Assamese, is a large prickly shrub, and its stem bark is ethnomedicinally prescribed in North-East India for treatment of various disease conditions like toothache, gingivitis, fever, colic vomiting, diarrhoea and cholera. Scientific parameters are not yet available to identify the exact plant material and to ascertain its quality and purity. The present investigation was therefore undertaken to determine the requisite pharmacognostical standards for evaluating the plant material. Various investigations like organoleptic or morphological characters, microscopic or anatomical studies, physico-chemical evaluations (loss on drying, ash values, extractive values), phytochemical screening, TLC finger print profiling and fluorescence analysis of powdered crude drug were carried out and the salient qualitative and quantitative parameters were reported. These studies provided referential information for correct identification and standardization of this plant material. These information will also be helpful to differentiate *Z. nitidum* from the closely related other species of *Zanthoxylum*.

Key words: *Zanthoxylum nitidum*, pharmacognostical, stem bark, quality control.

1. INTRODUCTION

The genus *Zanthoxylum* L. belongs to the family Rutaceae and is a large genus of aromatic prickly trees or shrubs distributed pan-tropically and 13 species of it are found in India. *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae), called *Tez-mui* in Assamese is a morphologically variable plant species occurring in south-east Asian countries and in Australia.[1] In India it grows as a large prickly shrub particularly in North-East India (Sikkim, Assam and Nagaland states). In India, the plant is traditionally used for various medicinal purposes. The root is used in toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide. The fruit is used in the treatment of stomachache, cough, colic vomiting, diarrhoea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seeds and stem bark are prescribed in fever, diarrhea and cholera.[2 - 4] It has come to our notice that the rural people of Assam use the stems and its bark as chewing material in treatment of toothache and gingivitis. However, no scientific standards or pharmacognostical parameters are yet available to ascertain the identity and to determine the quality of this crude drug. The pharmacognostical parameters are major and reliable criteria for confirmation of the identity and determination of quality and purity of the crude drugs. The present work therefore, attempts to report various necessary pharmacognostical standards of *Z. nitidum* stem bark.

2. MATERIALS AND METHODS

2.1. PLANT MATERIAL

The fully matured entire plants of *Z. nitidum* were collected during the month of November 2006 from Dibrugarh, Assam, India. The species was identified by Dr. S. J. Phukan, taxonomist, from Botanical Survey of India, Eastern Circle, Shillong, India, and a voucher specimen (No. DUPS-06-003) was deposited in Department of Pharmaceutical Sciences, Dibrugarh University for future reference. All the prickles were removed from the stems and branches carefully by using a sharp knife, without harming the bark. Then the barks were peeled off from the shoots. Longitudinal incisions were made by a sharp knife on the shoots and transverse markings were given so as to form the rings which also connect the longitudinal incisions producing the strips which were then peeled off. Then the stem barks were shade dried at temperature 21-24°C.

2.2. REAGENTS AND CHEMICALS

All reagents and chemicals used for testing were analytical grade obtained from Ranbaxy Fine Chemicals

Ltd., New Delhi and Loba Chemie, Mumbai, India. Brucine was obtained from Wilson Laboratories, Mumbai, India.

2.3. ORGANOLEPTIC EVALUATION

The freshly (just after collection) peeled stem bark of the plant were spreaded on a clean dry plastic sheet and investigated different organoleptic features by repeated observations using magnifying glass and ruler (where required) and recorded. Similarly the dried stem bark and root were also subjected to organoleptic evaluation.

2.4. MICROSCOPIC STUDIES

The transverse sections (TS) of stem bark were obtained by usual techniques.[5] Good sections were collected and placed on a grease free microscopic slide along with a drop of glycerin water (1:1). The sections were covered with clean cover slip and observed under the compound microscope at 40x magnification. A camera lucida was attached with the microscope and the sections were suitably traced out.[6]

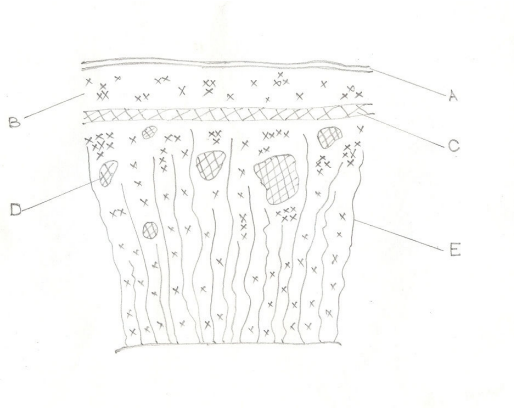

Figure 1. Schematic diagram, TS of the stem bark

A- cork, B- cortex, C- pericyclic sclerenchyma,
B- D- sclerenchyma E- medullary ray.

2.5. PHYSICO-CHEMICAL EVALUATIONS

Physico-chemical parameters such as the percentage of loss on drying (LOD), total ash, acid insoluble ash, water soluble ash were determined as per the Indian Pharmacopoeia.[7] Water and alcohol soluble extractives were estimated by hot extraction and cold maceration according to the method prescribed by WHO.[8] All determinations were performed in triplicate and the results are presented as mean ± standard error of mean (SEM).

2.6. PHYTOCHEMICAL SCREENING [9,10]

The dried and powdered stem bark was subjected to preliminary phytochemical screening for qualitative detection of phytoconstituents.

The dried and coarsely powdered stem bark (100 g) was extracted successively with petroleum ether (40-60ºC), chloroform (59.5-60ºC), ethyl acetate (76.5-77.5ºC), and ethanol (90%) in a soxhlet extractor by continuous hot percolation. Finally the marc was macerated with chloroform water. Each time before extracting with the next solvent of higher polarity the powdered drug (marc) was dried in a hot air oven below 50ºC for 10 minutes. Each extract was concentrated by distilling off the solvent, which was recovered subsequently. The concentrated extracts were evaporated to dryness and the extracts obtained with each solvent were weighed. Their percentages were calculated in terms of initial air dried plant material. The colours of extracts were observed.

The successive extracts, as mentioned above, were subjected to various qualitative phytochemical tests for the identification of chemical constituents present in the plant material.

2.7. THIN LAYER CHROMATOGRAPHIC STUDIES [11]

2.7.1 Preparation of bark extract (test solution): Approximately 1 g of dried coarsely powdered stem bark was taken in a 100 ml glass beaker and moistened with little amount (sufficient to moisten) of 25% liq. NH_3 with occasional stirring for 20 mins. Then the beaker was kept on boiling hot water bath to dry the contents for a few minutes .The beaker was cooled at room temperature and added 10-15 ml of chloroform and extracted on boiling hot water bath for 10 mins. Then the extract (1-2 ml) was collected in clean stoppered glass test tube and used for spotting the chromatographic plates.

2.7.2. Reference solution: A minute quantity of brucine (reference alkaloid) was dissolved in minimum amount (around 1 ml) of chloroform and kept in clean stoppered glass test tube and used as reference.

2.7.3. Stationary phase: Silica gel G, particle size 10-40µ, applied as a thin layer on a clean glass plate support and activated just before use.

2.7.4. Mobile phase: The mobile phase chosen was,

Toluene: Ethyl acetate: Diethylamine = 70: 20: 10.

2.7.5. Development method: One dimensional ascending method by using standard protocol as per

Indian Pharmacopoeia was followed.[7] The stem bark extract, prepared by above said method along with the reference alkaloid brucine solution, were chromatographed in the same plate.

2.7.6. Visualization: After development no visible spots were found. No spots were observed under UV light (short and long). Visualization was attempted by spraying with Dragendorff's reagent.

2.7.7. Documentation: After visualization by spraying with Dragendorff's reagent, dark and light orange brown spots were found. The R_f values of the spots were recorded carefully and the chromatogram was documented by graphical copying.[12]

2.8 FLUORESCENCE ANALYSIS [13,14]

A small quantity of dried and finely powdered stem bark was placed on a grease free clean microscopic slide and added 1-2 drops of the freshly prepared reagent solution, mixed by gentle tilting the slide and waited for 1-2 minutes. Then the slide was placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365 nm) ultraviolet radiations. The colours observed by application of different reagents in different radiations were recorded.

3. RESULTS

3.1. ORGANOLEPTIC EVALUATION: The characters recorded are described below.

3.1.1. Fresh bark

Condition: Moist.

Shape of pieces: Flat strips.

Dimensions: Varies, 8-12 cm. long, 1-2.5 cm wide and 0.2-0.4 cm thick.

Colour: Outer surface pale greenish brown, inner surface dull greenish yellow.

Odour: Aromatic, characteristic.

Taste: Aromatic and bitter. Proper chewing leads to typical pungent and worm sensation of tongue lasting for 10-15 minutes.

Fracture: Fibrous.

The outer surface of the bark had scattered lenticels and small and large scars left by the prickles and branches. Outer surface was marked by wavy longitudinal striations; inner surface also had longitudinal striations. The cork was found frequently exfoliated.

3.1.2. Dried bark

Condition: Hard and contracted.

Shape of pieces: Recurved and channeled quills.

Dimensions: Varies, 6-8 cm long and 0.4-0.8 cm wide.

Colour: Outer surface blackish brown, inner surface brownish buff coloured.

Odour: Slight.

Taste: Same as fresh bark.

Fracture: Splintery.

3.2. MICROSCOPIC STUDIES: The TS of bark is shown in Fig 1. The TS exhibited a cork consisting of narrow cells. The cortex contained small starch grains, crystals of calcium oxalate, but no sclereids. After cortex there was a narrow band of pericyclic sclerenchyma. The medullary rays were numerous, mainly one cell wide. Calcium oxalate crystals were also found in the phloem.

3.3. PHYTOCHEMICAL SCREENING: The results are shown in Table 1. The results demonstrated presence of true alkaloids, carbohydrates, flavonoids and amino acids in the stem bark of *Z. nitidum.* The percent extractives in different solvents and the colours of the extracts are summarized in Table 2.

3.4. PHYSICO-CHEMICAL EVALUATIONS: The values of all determinations are summarized in Table 3 & 4.Water soluble ash was found to be quite greater than acid insoluble ash value. The results showed greater extractive values (almost double) in hot extraction method. In both methods alcohol yielded higher extractives.

3.5. THIN LAYER CHROMATOGRAPHIC STUDIES: The stem bark extract yielded four orange-brown spots of different intensity, and the reference brucine showed one distinct spot. The results are shown in Table 5. The chromatogram is shown in Fig. 2.

3.6. FLUORESCENCE ANALYSIS: The results are summarized in Table 6.

4. DISCUSSION

To ensure reproducible quality of herbal products, proper control of starting material is utmost essential. Thus in recent years there has been an emphasis in standardization of medicinal plants of therapeutic potential. Despite the modern techniques, identification and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken. [8]

Organoleptic evaluation is a technique of qualitative evaluation based on the study of morphological and sensory profiles of whole drugs.[15] The organoleptic or macroscopic studies yielded important characteristics, such as the fractured surfaces of fresh and dried bark, typical tongue sensitizing aromatic taste and aromatic and characteristic odour of the bark; which are useful diagnostic characters. Similarly the microscopic or histological features, e.g. presence of pericyclic sclerenchyma, absence of sclereids, etc may be useful for this purpose.

The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. This is especially important for materials that absorb moisture easily or deteriorate quickly in presence of water. The test for loss on drying determines both water and volatile matter.[8,15]

The residue remaining after incineration of plant material is the ash content or ash value, which simply represents inorganic salts, naturally occurring in crude drug or adhering to it or deliberately added to it, as a form of adulteration. The ash value was determined by three different methods, which measured total ash, acid-insoluble ash, and water-soluble ash. The total ash method is employed to measure the total amount of material remaining after ignition. This includes both 'physiological ash' which is derived from the plant tissue itself, and 'non-physiological ash', which is the residue of the extraneous matter adhering to the plant surface. Acid-insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the water-soluble portion of the total ash**.** [6,15] These ash values are important quantitative standards.

The extracts obtained by exhausting plant materials with specific solvents are indicative of approximate measures of their chemical constituents extracted with those solvents from a specific amount of air-dried plant material. This parameter is employed for materials for which as yet no suitable chemical or biological assay exist.[7,8] The results showed greater extractive values (almost double) in hot extraction, indicating the effect of elevated temperature on extraction.[17] In all methods alcohol yielded higher extractives.

The plant material was subjected to preliminary phytochemical screening involving successive solvent extraction by different solvents in order of increasing polarity to obtain diverse polar and non polar phytoconstituents possessing different solubility pattern, followed by various chemical tests for qualitative detection of various chemical constituents.[9,15] And it was found that true alkaloids and flavonoids are present in ethanol extract and carbohydrates and amino acids are in aqueous extract. Alkaloid content was found to be appreciable as compared with other constituents. The percent extractives in different solvents indicate the quantity and nature of constituents in the extract. The colour of the extract sometimes may roughly indicate the physical and chemical features of constituents present.

Thin layer chromatography (TLC) is particularly valuable for the preliminary separation and determination of plant constituents. As per phytochemical screening the stem bark of *Z. nitidum* contains mainly true alkaloids. The standard alkaloid used was brucine with known R_f value of 0.253 with same solvent system to assess the accuracy of the study.[11] The visualizing reagent employed was Dragendorff's reagent to effect visualization of the resolved spots which were invisible otherwise (visibly and under UV radiations). The bark extract yielded four orange-brown spots with different intensities, and brucine showed one distinct spot of R_f value 0.30 which however, did not comply with that of literature (mentioned above). In fact, it is very difficult to reproduce the experimental conditions of TLC and hence, the obtained R_f value differed to some extent from that of literature. The chromatographic profile may serve as a characteristic finger print for qualitative evaluation of bark.

Fluorescence is the phenomenon exhibited by various chemical constituents present in the plant material. Some constituents show fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products (e.g. alkaloids like berberine), which do not visibly fluoresce in daylight. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents. Hence, some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation.[14,18]

After present investigation it can be concluded that the pharmacognostical study of *Z. nitidum* stem bark yielded a set of qualitative and quantitative parameters or standards that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material in future studies. As previously mentioned, *Z. nitidum* being a morphologically variable species, these information will also be helpful to differentiate *Z. nitidum* from the closely related other species and varieties of *Zanthoxylum.*

Table 1. Results of phytochemical screenings of successive extracts of stem bark of *Z. nitidum*

Constituents	Pet. Ether extract	Chloroform extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
Alkaloids	-	-	-	+	-
Purine alkaloids	-	-	-	-	-
Carbohydrates	-	-	-	+	+
Glycosides	-	-	-	-	-
Steroids	-	-	-	-	-
Flavonoids	-	-	-	+	-
Saponins	-	-	-	-	-
Fixed oils and fats	-	-	-	-	-
Tannins	-	-	-	-	-
Proteins and amino acids	-	-	-	-	+
Mucilage	-	-	-	-	-

+ = Present, – = Absent.

Table 2. Percent extractives and colours of successive extracts of *Z. nitidum* stem bark

Solvent	Extractive values (% w/w)	Colours of extracts
Pet. Ether.	1.57	Shining yellow
Chloroform	2.81	Deep greenish black
Ethyl acetate	1.75	Light green
Ethanol	9.13	Greenish yellow
Water	6.85	Black

Table 3. Loss on drying (LOD) and ash values of powdered stem bark of *Z. nitidum*

Parameters	Values of three replicates (% w/w)	Mean (% w/w) ± SEM
Loss on drying (LOD)	4.17 6.20 7.19	5.85 ± 0.89
Ash values: 1) Total ash	5.80 6.00 5.62	5.81 ± 0.11
2)Acid insoluble ash	0.90 0.75 0.79	0.81 ± 0.05
3)Water soluble ash	3.79 3.94 3.72	3.82 ± 0.06

SEM = Standard Error of Mean

Table 4. Extractive values of stem bark of *Z. nitidum*

Method of extraction	Values of three replicates (% w/w)	Mean (% w/w) ± SEM
Cold maceration: 1) Water soluble	2.32 2.96 2.52	2.62 ± 0.18
2) Alcohol soluble	3.73 3.84 3.36	3.73 ± 0.10
Hot Extraction: 1) Water soluble	3.96 4.36 4.21	4.18 ± 0.12
2) Alcohol soluble	5.87 6.32 6.13	6.12 ± 0.12

SEM = Standard Error of Mean.

Table 5. Observations of thin layer chromatographic studies of stem bark of *Z. nitidum*

Extracts	Mobile phase	No. of spots	R_f. values	hR_f. values	Intensity
Stem bark	Toluene: Ethyl acetate: Diethylamine = 70: 20: 10	4	0.22 0.44 0.70 0.90	22 44 70 90	+ ++ +++ ++
Brucine (Reference)	Do	1	0.30	30	++

Visualizing reagent: Dragendorff's reagent. +++ = Most intense, ++ = Moderately intense, + = Least intense

Table 6. Fluorescence analysis of powdered stem bark of *Z. nitidum*

Powdered drug	Visible/Day light	UV 254 nm (short)	UV 365 nm (long)
Powder as such	Light yellowish brown	Brown	Blackish brown
Powder + 1M NaOH	Yellowish brown	Dark yellowish brown	Dark brown
Powder +1% Picric acid	Yellowish brown	Brown	Black
Powder + Acetic acid	Brown	Dark brown	Blackish brown
Powder + 1M HCl	Brownish yellow	Brown	Dark brown
Powder + Dil HNO_3	Brownish yellow	Brown	Dark brown
Powder + 5% Iodine	Yellowish brown	Dark brown	Black
Powder + 5% $FeCl_3$	Yellowish brown	Brown	Black
Powder + HNO_3+ 25 % NH_3	Yellowish brown	Yellow	Black
Powder + Methanol	Yellowish brown	Dark brown	Blackish brown
Powder + 50% HNO_3	Yellowish brown	Brown	Dark brown
Powder + 1M H_2SO_4	Brown	Dark brown	Blackish brown
Powder + Dil. NH_3	Yellowish brown	Light brown	Brown
Powder + Conc. HNO_3	Yellowish brown	Brown	Dark brown
Powder +10% Potassium dichromate soln	Deep yellow	Yellowish brown	Black
Powder + 25% Liquid NH_3	Yellowish brown	Brown	Blackish brown

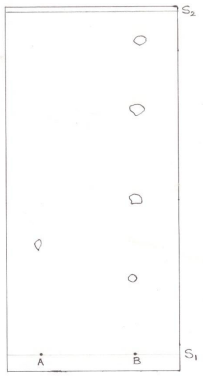

Figure 2. The chromatogram
A – Brucine (reference), B- Stem bark extract of *Z. nitidum*
S_1 - Sample application point,S_2 – Solvent front

5. ACKNOWLEDGEMENT

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